

CLAIMS:

1. A hybrid plasminogen activator comprising a polypeptide bond union between streptokinase (SK), or modified forms of SK, or suitable parts thereof, which are capable of plasminogen (PG) activation, with fibrin binding regions of human fibronectin selected from the pair of fibrin binding domains 4 and 5, or domains 1 and 2, or modified forms thereof, to achieve various motifs for joining the fibrin binding domains with streptokinase or its modified forms, so that the hybrid plasminogen activator possesses the ability to bind with fibrin independently and also characteristically retains a plasminogen activation ability which becomes evident only after a pronounced duration, or lag, after exposure of the plasminogen activator to a suitable animal or human plasminogen.

2. A hybrid plasminogen activator as claimed in Claim 1 wherein the streptokinase molecule bind with the fibrin binding regions of human fibronectin selected from the pair of fibrin binding domains 4 and 5, domains 1 and 2 or modifications thereof as depicted in Figure 1.

3. A hybrid plasminogen activator as claimed in Claim 1 which carries out plasminogen activation only after a lag period varying between 5 and 30 minutes after exposure of the plasminogen activator to a suitable animal or human plasminogen

4. A DNA segment encoding the hybrid plasminogen activator as claimed in Claims 1 and 2.

5. An expression vector containing a DNA segment as claimed in Claim 3.

6. Prokaryotic or eukaryotic cells, transformed or transfected with expression vectors as claimed in Claim 4 and capable of expressing the hybrid plasminogen activators as claimed in Claim 1.

7. A method for the preparation of hybrid plasminogen activator exhibiting plasminogen activation characteristics, said method comprising the steps of :

(a) Preparing a first DNA encoding a nucleotide sequence for streptokinase or any of its modified forms, by conventional biochemical or chemical methods or appropriate combinations thereof, to produce a translational product which is a polypeptide that can activate plasminogen

- (b) Preparing a second DNA polynucleotide by known biochemical or chemical methods or appropriate combinations thereof, that encodes for the fibrin binding domains selected from the pair of fibrin binding domains 4 and 5, or domains 1 and 2, or their modified forms, that are capable of conferring affinity and/or specificity for fibrin, and linking these to another DNA molecule that is capable of undergoing multiplication in a suitable host cell,
- (c) Construction of hybrid polynucleotides, between the first DNA encoding for streptokinase, or its modified forms, that encode for a polypeptide capable of plasminogen activation, with the second DNA encoding for the fibrin binding domains (FBD) of fibronectin by conventional methods, in the native translational codon frame starting with an initiator codon, and joining of the hybrid polynucleotide into a recipient DNA molecule, such as a plasmid capable of autonomous replication in a host cell, or capable of integrating into the genomic DNA of a suitable host cell, and expressing the hybrid protein therein,
- (d) Introducing the DNA containing the hybrid polynucleotide constructs obtained in step (c) into an appropriate host, selected from the group comprising *E. coli*, *Bacillus sp.*, yeast, fungus, plant, animal cell by conventional methods,
- (e) Culturing the host cells expressing the SK-FBD chimeric polynucleotide using known procedures,
- (f) Isolating components of the culture, selected from extracellular fluid from fermentation, intracellular milieu of the host cell or combinations thereof, that harbour the expressed chimeric polypeptide in an enriched form, and then partially purifying the chimeric polypeptides using conventional procedures selected from the group comprising centrifugation, ultrafiltration, precipitation with salts or organic solvents etc, or chromatography on suitable media, or combinations thereof,
- (g) Refolding the hybrid polypeptide to a biologically active and structurally intact form, if required,
- (h) Further purifying the biologically active hybrid polypeptide from the relatively crude or partially pure material/s or cell-free lysate obtained at step (f), or (g) above, after refolding, using conventional methods of protein purification, or by

affinity chromatography on a suitable matrix comprising immobilized fibrin or fibrinogen, or specific antibodies that recognize and bind with the active, biologically active hybrid proteins,

8. A method as claimed in claim 7 which includes DNA segments/polynucleotide blocks encoding the polypeptides, plasmids containing these genetic elements capable of their expression into protein, as well as microorganisms or other suitable host cells transformed with these plasmids.

9. A method as claimed in claim 7 in pure and biologically active form for clinical and research applications.

10. A method as claimed in claim 7 wherein large quantities of streptokinase or modifications thereof are produced using an altered polynucleotide block encoding for streptokinase or its modified forms, and obtain these in a pure and biologically active form.

11. A method as claimed in claim 7 wherein the 5'-end of the streptokinase encoding polynucleotide utilized for expression of streptokinase or its modified forms such as the streptokinase FBD chimeric polypeptides, is modified, as exemplified by the DNA sequence provided in Fig. 13, by conventional methods such as mutagenesis, biochemical or chemical DNA synthesis techniques, or their combinations, such that the secondary structure-forming capability (e.g., the intramolecular hydrogen bonding capability) of its transcript is diminished, resulting in increased efficiencies of expression of SK or its modified forms such as SK-FBD chimeras in the heterologous host cell.

12. A method as claimed in claim 7 wherein the 5'-end of the SK-encoding DNA or its modified forms such as the SK-FBD chimeric polypeptides, is modified by mutagenesis by known biochemical or chemical DNA synthesis techniques, or a suitable combination thereof, in such manner that the codons utilized in the DNA polynucleotide are compatible with those frequently utilized in *E. coli* or the host cell used for the expression of the genes.

13. A method as claimed in claim 7 wherein the DNA encoding those fibrin binding domains that possess independent fibrin binding capability are fused in the correct translational frame at the 5'-end of the SK-encoding DNA, after a translational start codon, and then expressed into protein of the form exemplified in Fig. 1C, refolded oxidatively and isolated in the purified form, to obtain the desired characteristic in the chimera viz.,

characteristic PG activation properties characterized by an initial lag in the PG activation rates together with significant fibrin affinity.

14. A method as claimed in claim 7 wherein the fibrin binding domains are fused in-frame at the C-terminal end of the SK, or its modified form, to obtain a hybrid SK-fibrin binding domain protein that contains selected fibrin binding domains at the C-terminal end of the SK portion of the chimera after expression of the hybrid DNA in a suitable host cell, as exemplified in Figs. 1A and 1B, to obtain the desired characteristic in the chimera viz., characteristic PG activation properties characterized by an initial lag in the PG activation rates together with significant fibrin affinity.

15. A method as claimed in claim 7 wherein the fibrin binding domains are fused through polypeptide linkage at the C-terminal end of the SK devoid of upto 45 amino acids, preferably 31 amino acid residues. Thus, a hybrid SK-FBD protein is generated that contains selected fibrin binding domains fused at the C-terminal end of a truncated SK, thus yielding a chimeric protein that has both fibrin affinity as well as delayed PG activation properties.

16. A method as claimed in claim 7 wherein fibrin binding domains are fused at both the ends of SK, or its modified forms that retain a plasminogen activator ability, simultaneously (in the configuration represented as 'FBD-SK-FBD'; as schematically depicted in Fig. 1, D) to achieve the desired functionality in the hybrid construct viz., characteristic plasminogen activation properties characterized by an initial lag in the PG activation rates together with significant fibrin affinity.

17. A method as claimed in claim 7 wherein the novel chimeric polypeptides are expressed in *E. coli* or other suitable host cells.

18. A process as claimed in claim 7 wherein SK or its truncated form/s are fused through polypeptide linkages with the fibrin binding domains known to possess independent fibrin binding capability through a short linker peptide region comprising of a stretch of amino acid sequence that is not conformationally rigid but is flexible, such as those predominantly composed of Gly, Ser, Asn, Gln and the like amino acids.

19. A method as claimed in claim 7 wherein SK or its modified forms are fused with fibrin binding domains through a "linker" peptide composed of amino acid sequences that provide varying levels of local conformational flexibility by incorporating sequences that fold into relatively rigid secondary structures such as beta-turns so as to obtain different

chimeric PG activator proteins with desirable initial lag-times in plasminogen activation kinetics.

20. A method as claimed in claim 7 wherein the host *E. coli* cells are lysed by chemical treatment such as chaotropic salts e.g., guanidinium hydrochloride and the like, to effect the liberation of the SK or its modified chimeric forms, followed by purification using conventional methods.

21. A method as claimed in claim 7 wherein the crude cell-lysates obtained, using either conventional methods or by employing chaotropic salts, from cells elaborating the chimeric polypeptides are subjected to air oxidation to refold the chimeric polypeptides to their biologically active conformations containing the native cystine connectivities.

22. A method as claimed in claim 7 wherein the crude cell-lysates obtained using either conventional methods selected from the group consisting of enzymatic lysis of cells, ultrasonic lysis, lysis by mechanical means or by employing chaotropic salts, from cells elaborating the chimeric polypeptides are subjected to oxidation and refolding using a mixture of reduced and oxidized glutathione of a suitable redox potential that allows the chimeric polypeptides to refold to their biologically active conformations.

23. A method as claimed in claim 7 wherein the refolding reaction is carried out in the presence of immobilized fibrin to promote a more efficient ligand-induced refolding of the epitopes responsible for fibrin affinity in the said chimeric polypeptides, and consequently higher yields of the biologically active chimeric protein constructs.

24. A method as claimed in claim 7 wherein the biologically active chimeric polypeptides are purified selectively from other proteins, or unfolded SK-FBD polypeptides, by affinity chromatography on immobilized fibrin(ogen) e.g. fibrin- or fibrinogen-agarose.

25. A method as claimed in claim 7 wherein the SK-FBD hybrid polypeptides are expressed in *E. coli* using known plasmids under the control of strong promoters, such as *tac*, *trc*, *trp*, T7 RNA polymerase and the like, which also contain other well known features necessary to effect high level expression of the incorporated DNA polynucleotides encoding for the hybrid Streptokinase-fibrin binding domain polypeptides e.g. Shine-Delgarno sequence, transcription terminating signals etc.

26. A method as claimed in claim 7 wherein SK or its truncated forms are fused either at the amino- or C-termini, or both, through polypeptide linkages with the FBDs known to possess independent fibrin binding capability, such as domains 4 and 5, through short 'linker' regions, as described above, that contain amino acid sequence/s providing varying levels of local conformational flexibility to the linker segment between the SK and FBD portions of the hybrid protein/s.

27. A method as claimed in claim 7 wherein various chemical or physical agents, such as iso-propyl- beta-D-thio galacto pyranoside (IPTG), lactose, low or high temperature change, change in salt or pH of medium, ethanol, methanol, and the like, are used to induce high levels of the SK or the various hybrid polypeptides in the host cell in which the hybrid polynucleotides are being expressed.

28. A method as claimed in claim 7 wherein the hybrid SK-FBD polynucleotides are expressed in *E. coli*.

29. A method as claimed in claim 7 wherein the *E. coli* cells are lysed by chemical treatment such as the use of chaotropic salts e.g. guanidinium hydrochloride and the like, to effect the liberation of the SK or its modified hybrid forms, which are then purified using conventional procedures.

30. A method as claimed in claim 7 wherein the host *E. coli* cells are lysed by chemical treatment such as chaotropic salts e.g., guanidinium hydrochloride and the like, to effect the liberation of the SK or its modified chimeric forms, followed by purification using conventional methods.

31. A method as claimed in claim 7 wherein a chimeric plasminogen activator protein is used as a medicant for the treatment or prophylaxis of thrombolytic diseases; the said activator may be formulated in accordance with routine procedures as pharmaceutical composition adapted for intravenous administration to human beings, and may contain stabilizers such as human serum albumin, mannitol etc, solubilizing agents, or anesthetic agents such as lignocaine and the like.

32. A pharmaceutical composition comprising a hybrid plasminogen activator and stabilizers such as human serum albumin, mannitol etc, solubilizing agents, anesthetic agents.